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ORIGINAL RESEARCH

Potential Pathogenetic Role of Antimicrobial Peptides Carried by Extracellular Vesicles in an in vitro Psoriatic Model

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Purpose: Extracellular Vesicles (EVs) are a heterogeneous group of cell-derived membranous nanoparticles involved in several physiopathological processes. EVs play a crucial role in the definition of the extracellular microenvironment through the transfer of their cargo. Psoriasis is a prototypical chronic inflammatory disease characterized by several secreted mediators, among which antimicrobial peptides (AMPs) are considered pivotal in the development of the psoriatic inflammatory microenvironment. The role of EVs in the pathogenesis of psoriasis has not been elucidated yet, even if emerging evidence demonstrated that interleukin-17A (IL-17A), the psoriasis-related principal cytokine, modifies EVs release and cargo content. The aim of this work was to analyze whether, besides IL-17A, other psoriasis-related cytokines (ie, IFN-γ, TNF-α, IL-22 and IL-23) could affect EVs release and their AMPs mRNAs cargo as well as to analyze the potential biological effect due to EVs internalization by different acceptor cells.

Methods: Nanoparticle tracking analysis (NTA) was performed on supernatants of HaCaT cells stimulated with IL-17A, IFN-γ, TNF-α, IL-22 or IL-23 to enumerate EVs. Real-Time RT-PCR was used for gene expression analysis in cells and EVs. Confocal microscopy and Flow cytometry were used to, respectively, study Netosis and EVs internalization.

Results: IL-17A and IFN-γ increased EVs release by HaCaT cells. All the tested cytokines modulated AMPs mRNA expression in parental cells and in their respective EVs. *S100A12* and *hBD2* mRNAs were upregulated following IL-17A and IL-22 treatments. Interestingly, EVs derived from cytokine treated HaCaT cells induced Netosis in freshly isolated neutrophils. Upregulation of *S100A12* and *hBD2* mRNA was also detectable in acceptor cells incubated with EVs derived from cells treated with psoriasis-related cytokines.

Conclusion: The obtained results highlighted the role of EVs in the composition of psoriasis-associated secretome and microenvironment also suggesting the EV involvement in the spreading of the disease mediators and in the possible associated comorbidities.

Keywords: extracellular vesicles, antimicrobial peptides, cytokines, keratinocytes, pathogenesis of psoriasis, comorbidities

Introduction

Psoriasis vulgaris is a chronic immune-mediated inflammatory skin disease affecting approximately 2.0% to 3.0% of the world's population.[1] It is characterized by dynamic dermatosis and it can produce various comorbidities during the evolution of an individual lesion.[2] Even if the pathogenesis of psoriasis has not been completely clarified yet, several reports showed that, after an initial external insult, two important events occur to activate the innate immune system: (i) dramatical secretion of specific antimicrobial peptides (AMPs) and (ii) release of DNA, RNA and other endogenous danger signals.[3] The subsequent activation of the adaptive immune response, via a pathogenic cross talk between keratinocytes, dendritic cells (DCs) and T cells, drives the maintenance phase of psoriatic inflammation. In particular, Th17 cells produce IL-17A while cytotoxic $CD8^+$ T lymphocytes migrating to the lesional psoriatic skin act as a source

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of IL-17A, tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ) and IL-22 in the epidermis.[4,5] Moreover, inflammatory DCs produce IL-23, nitric oxide (NO) and TNF-α.[6]

Antimicrobial peptides are 12–50 amino acid residues positively charged peptides with an amphipathic structure, synthesized by both commensal skin microbiota, keratinocytes and neutrophils.[7] In addition to their direct antimicrobial function, AMPs play an important additional role in the orchestration of the immune response. Cationic 18-kDa pre-pro-protein (hCAP-18) is secreted and processed by serine proteases into its active form, called LL-37. LL-37 can facilitate immune-activating functions, such as chemokine production, leukocyte recruitment, macrophage and DC differentiation as well as T-cell polarization but can also mediate anti-inflammatory responses.[8] LL-37 has been hypothesized to drive inflammation in psoriasis through its capability to enable plasmacytoid DCs (pDCs) to recognize a LL-37/DNA complex through TLR9.[9,10] Defensins are other members of AMP family. Human defensin-β (hBD)2 and hBD3 expression is upregulated following stimulation by microorganisms and microbial components (such as bacterial lipopolysaccharides, LPS) or by pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-17A and IL-22.[11] hBD2, hBD3, and hBD4 stimulate keratinocytes to produce pro-inflammatory cytokines and chemokines such as IL-6, IFNγ-induced Protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-3α (MIP-3α) and regulated on activation normal T cell expressed and secreted (RANTES).[12] hBD2 systemic levels are strongly correlated with psoriasis area and severity index (PASI) score.[13] S100 proteins are a family of low molecular weight (9–13-kDa) proteins characterized by the presence of two calcium binding sites of helix–loop–helix motifs with damage-associated molecular patterns (DAMPs) characteristic.[14] In particular, S100A12 can also be detected in endothelial cells, keratinocytes, epithelial cells and pro-inflammatory macrophages under inflammatory conditions.[15] In psoriatic patients, there is a significant correlation between PASI and S100A12 serum levels.[16]

Extracellular vesicles (EVs) are a heterogeneous family of membrane-limited nanoparticles generated from different subcellular compartments and released into the extracellular space, even if they can also reach systemic circulation. They are classified into three subtypes based on their biogenesis and size: exosomes (Exos), microvesicles (MVs) and apoptotic bodies (ABs). EVs embed molecules such as DNA, mRNA, microRNA (miRNA) and proteins as a result of a yet incompletely understood regulated sorting mechanism into target cells.[17,18] EVs-based intercellular communication is involved in several physiological, as well as pathological, processes and may trigger or suppress the immune response. Additionally, EVs and can be used as potential biomarkers or engineered drug carrier in inflammatory skin disorders.[19,20] Emerging evidence demonstrates that IL-17A affects the release and hBD2 cargo of keratinocytes derived EVs,[21] but no data describing the role of the most important psoriasis-related cytokines (ie, IFN-γ, TNF-α, IL-17A, IL-22 and IL-23) in EV modulation are available yet.

Here, we show how psoriasis-related cytokines can differentially affect the release of EVs by a stabilized human keratinocyte cell line (ie, HaCaT cells) and modulate the AMPs mRNAs expression into EVs cargo. In addition, we suggest a role of EVs into intercellular communication in the inflammatory microenvironment, in the spreading of the disease and in the keratinocyte motility, then contributing to the hypercellularity typical of the psoriatic lesion, as well as in the establishment of psoriasis-associated comorbidities. In this respect, we report results about the ability of EVs, derived from HaCaT stimulated with psoriasis-related cytokines, i) to induce Netosis in in vitro treated freshly isolated neutrophils and ii) to be heterotopically internalized by SW-1356 and SW-982 cells (ie, a human chondro-sarcoma- and a synovial sarcoma-derived cell lines), thereby affecting *hBD2, hCAP18* and *S100A12* gene expression.

Materials and Methods

Cell Cultures and Reagents

The spontaneously transformed aneuploid immortal keratinocyte HaCaT cell line was purchased from Cell Line Service GmBH and was cultured in Dulbecco's Modified Essential Medium (DMEM, Corning, New York, USA), 4.5 g/L glucose at 37°C and 5.0% CO_2. Chondrosarcoma (SW-1353) and synovial sarcoma (SW-982) human cell lines were purchased from ATCC and were grown in Leibovitz's (L-15) Medium (Corning) at 37°C. Both L-15 and DMEM media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 UI/mL penicillin and

100 μg/mL streptomycin (Corning). Heat-inactivated, exosome-depleted FBS was obtained by ultracentrifugation at 100000x g for 18 hours, following by filtration using 0.1 μm PES filter (Sartorius, Gottinga, Germany).

Recombinant (r)IFN-γ, rTNF-α, rIL-17A, rIL-22 and rIL-23 were all purchased from PeproTech (Cranbury, NJ, USA), and used unless otherwise reported at final concentration of 100 ng/mL.

PKH67 Green Fluorescent Cell Linker MidiKit for General Cell Membrane Labeling was purchased from Sigma-Aldrich/Merck (St. Louis, MO, USA).

Nanoparticle Tracking Analysis (NTA)

Supernatants from HaCaT cells, treated with recombinant cytokines for 72 hours in medium supplemented with Exosome-depleted FBS, were centrifuged 500 × g for 10 minutes then 2000 × g for 10 minutes to remove large cellular debris. Subsequently, each sample was properly diluted in 0.1 μm filtered PBS, acquired using a NanoSight NS300 instrument (Malvern Panalytical, Malvern, UK) (NTA) and analyzed by Nanosight v.3.4 software.

RNA Isolation and Real Time RT-PCR on Cells and EVs

HaCaT cells were seeded in 175 cm^2 flasks and stimulated as described above. Supernatants were collected and EVs were isolated as previously described, whereas cells were washed twice in PBS. SW982 and SW1353 cells were seeded into 6-well plate and, after 24 hours, pulsed with EVs derived from untreated HaCaT cells as well as stimulated with psoriasis-related cytokines for 3 and 24 hours. Total RNA was extracted from cells and HaCaT derived EVs using the Total RNA Purification Kit (Norgen Biotek Corp., Ontario, Canada). One μg of total RNA was retro-transcribed using the Tetro cDNA synthesis kit (Meridian BioScience, Cincinnati, Ohio, USA), and cDNA products were analyzed by Real-Time RT-PCR using the SensiMix SYBR Hi-ROX Kit (Meridian Bioscience, Cincinnati, OH, USA). Data were normalized using as endogenous controls HPRT-1, or 18S rRNA for EV data, and expressed using the $2^{-\Delta\Delta CT}$ method.[22] The gene-specific primers used to detect AMPs mRNAs are listed in Supplementary Table 1.[21,23,24]

EVs Internalization Assay

HaCaT cells were seeded in a 175 cm^2 flask in DMEM, 10% Exosome-depleted FBS. EVs were collected as previously described and stained with the PHK67 green fluorescent dye, following the manufacturer's instructions. Subsequently, HaCaT, SW1353 and SW982 acceptor cells were incubated at 1:1 v/v ratio with PHK67-labelled HaCaT-derived EVs in DMEM or L-15 medium, both supplemented with 10% Exosome-depleted FBS, at 37°C for 0.5, 1, 3, 6, 18 hours or at 4°C for 6 hours, as a negative control.[25] EVs uptake by acceptor cells was evaluated by flow cytometry using a FACS ARIA II Cell Sorter equipped with FACS DiVa software v6.1.1 (Becton Dickinson, Franklin Lakes, NJ, USA). Results were analyzed using Flowing software v2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland).

Netosis Induction and Confocal Microscopy

Neutrophils were isolated from peripheral blood of healthy donors by centrifugation on a Polymorphprep (Progen, Heidelberg, Germany) density gradient. Isolated neutrophils were seeded at $3x10^4$/point on coverslips pretreated with poly-lysine and stimulated 3 hours with EVs obtained with HaCaT cells treated with inflammatory cytokines as previously described or with PMA (Sigma Aldrich, 50 ng/mL) as positive control. After stimulation, cells were gently washed, fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin (Sigma Aldrich) in the presence of 1% of normal goat serum (Jackson Laboratories, Bar Harbor, ME, USA). To visualize NETs, cells were stained 30 minutes with rabbit anti-human LL37 (1 μg/mL; Innovagen, Lund, Sweden), washed and stained with an $AlexaFluor^{488}$-conjugated goat anti-rabbit (Abcam, Cambridge, UK). DNA filaments were stained with a DNA dye (DAPI). Slides were mounted with Prolong Gold antifade media (Molecular Probes/Thermofisher, Waltham, MA, USA). Images were acquired by a confocal microscopy Zeiss LSM900 (Carl Zeiss GmbH, Jena, Germany). Optical thickness varies according to objective used from 0.5 μm with 20x objective to 0.2 μm with 63x objective. The level of netosis was calculated by measuring the DNA occupied area (DAPI) of both the nucleus and the extracellular strands and subtracting the mean value of the area of the nuclei in the control group (neutrophils stimulated with medium). Area values were measured by Zen Blue (3.2) software (Carl Zeiss GmbH). Images have been treated by ImageJ (NIH, USA).

Migration Assay

Transwell migration assay was carried out as previously described,[26] with some modifications. Briefly, HaCat cells were harvested, resuspended in DMEM medium supplemented with 10% exosome-depleted FBS, and seeded in 24-well cell culture inserts with 8 μm pore size membrane (Boyden chambers, Becton Dickinson) at 2×10^5 cells/insert. The lower chamber was, then, filled with DMEM medium supplemented with 10% Exosome-depleted FBS, which was added with EVs isolated from HaCat cells untreated, treated with rIL-17 or with rIFN-γ. Cells were incubated at 37°C for 18 hours allowing migration across the membrane pores. At the end of incubation, cells were fixed with 100% methanol and non-migrated cells were removed from the inner side of the insert with a cotton swab. The lower surface of the membrane was gently rinsed with PBS, stained for 15 min with Giemsa staining solution (Sigma Aldrich) diluted 1:20 in water, rinsed again and allowed to dry at room temperature. Five random fields per insert were photographed with a light microscope (Leica DM 4000B, Leica Biosystems, Wetzlar, Germany) at 10× magnification and ImageJ software (NIH, USA) was used for cell count.

Statistical Analysis

Statistical analyses were performed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test or Wilcoxon matched pairs signed rank test, using GraphPad PRISM software v5.0 (GraphPad Software, Inc., San Diego, CA, USA). $P \leq 0.001$ (***); $P \leq 0.01$ (**); $P \leq 0.05$ (*). Error bars in the graphs represent the standard deviation.

Results

Psoriasis-Related Cytokines Treatments Modify HaCaT Derived Extracellular Vesicles Release

Previous results obtained by our group demonstrated the ability of IL-17A to quantitatively and qualitatively affect the EVs release of human keratinocytes.[21] To verify if other psoriasis-related cytokines could modify the release of EVs by keratinocytes, HaCaT cells were treated with rIFN-γ, rTNF-α, rIL-17A, rIL-22 and rIL-23 for 72 hours, then the EVs isolated from the supernatant were evaluated by Nanoparticle Tracking Analysis (NTA) analysis. Figure 1 shows that only IL-17A (Figure 1B) and IFN-γ (Figure 1F) led to an increased release of EVs. Regardless of the treating cytokine, most of the released EVs had a diameter less than 200 nm, compatible with small EVs size (for more information about counts and mean size see Supplementary Table 2).

mRNA Expression of hBD2 and hS100A12 Was Strongly Upregulated by rIL-17A and rIL-23 in HaCaT Cells and Related EVs

It was established that rIL-17A treatment allows the release of EVs containing a set of specific mRNAs, in particular *hBD2* mRNA.[21] To evaluate whether other psoriasis-related cytokines could modulate the expression of antimicrobial peptides mRNAs loaded into EVs, HaCaT cells were treated with rIFN-γ, rTNF-α, rIL-17A, rIL-22 and rIL-23 for 72 hours. Following the treatments, the expression levels of *hCAP-18, hBD2, hBD4* and *hS100A12* into cells and EVs were evaluated by Real-Time RT-PCR (Figure 2). rIL-17A and rIL-23 cell treatment induced a statistically significant increase of *hBD2* and *hS100A12* mRNAs (Figure 2A and B). Similarly, rIL-17A and rIL-23 caused a statistically significant increase of *hBD2* mRNA into EVs (Figure 2D). On the other hand, in EVs, rIL-17A, rTNF-α, r-IL-22 and rIL-23 induced the increase of *hS100A12* mRNA (Figure 2E). *hCAP-18* mRNA was not found to be modulated by all the treatments either in HaCaT cells or in their EVs (Figure 2C and F).

EVs Isolated from Cytokine Treated HaCaT Cells are Able to Induce NETosis in vitro

Neutrophil extracellular traps (NETs) have been implicated in the development of certain immune-mediated diseases among which psoriasis.[27] NETs formation is the consequence of a neutrophils-mediated cell death process defined NETosis that contributes to local inflammation.[28] It has been reported that keratinocyte-derived exosomes activate neutrophils via NF-κB and p38 MAPK and that in psoriatic skin cationic antimicrobial peptides cooperate to break tolerance.[29–31] To verify the hypothesis that, besides cationic peptides, EVs could modify the psoriatic microenvironment

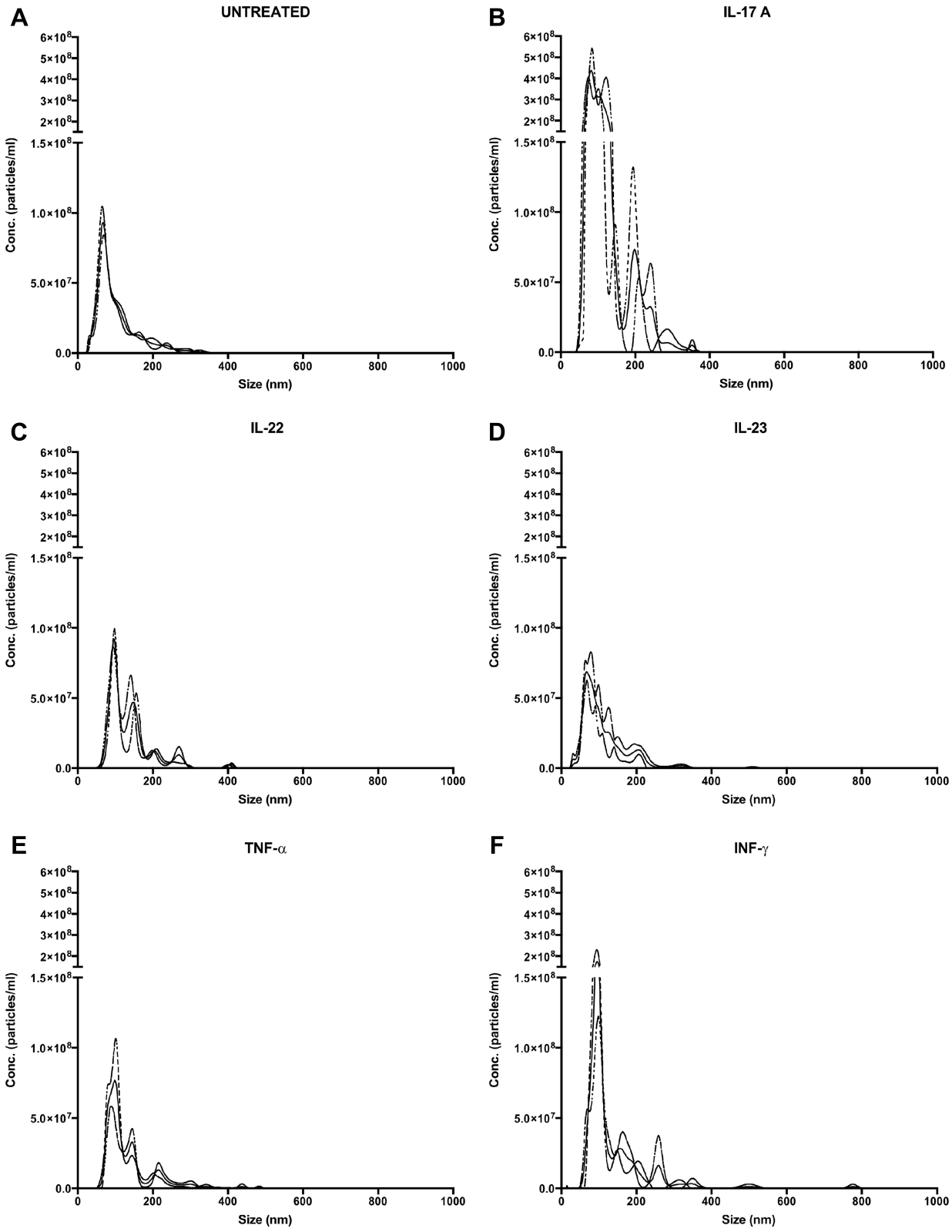

Figure 1 rIL-17A and rIFN-γ treatment of HaCaT cells increase the release of small EVs. EVs were quantified by NTA from supernatants collected from untreated Untreated HaCaT cells (**A**), or treated with rIL-17A (**B**), rIL-22 (**C**), rIL-23 (**D**), rTNF-α (**E**) and rIFN-γ (**F**) for 72 hours as described in Materials and Methods. Each sample was acquired three times at 22.2–22.3°C. The solid line represents the acquisitions mean while the dashed lines represent ± standard deviations.

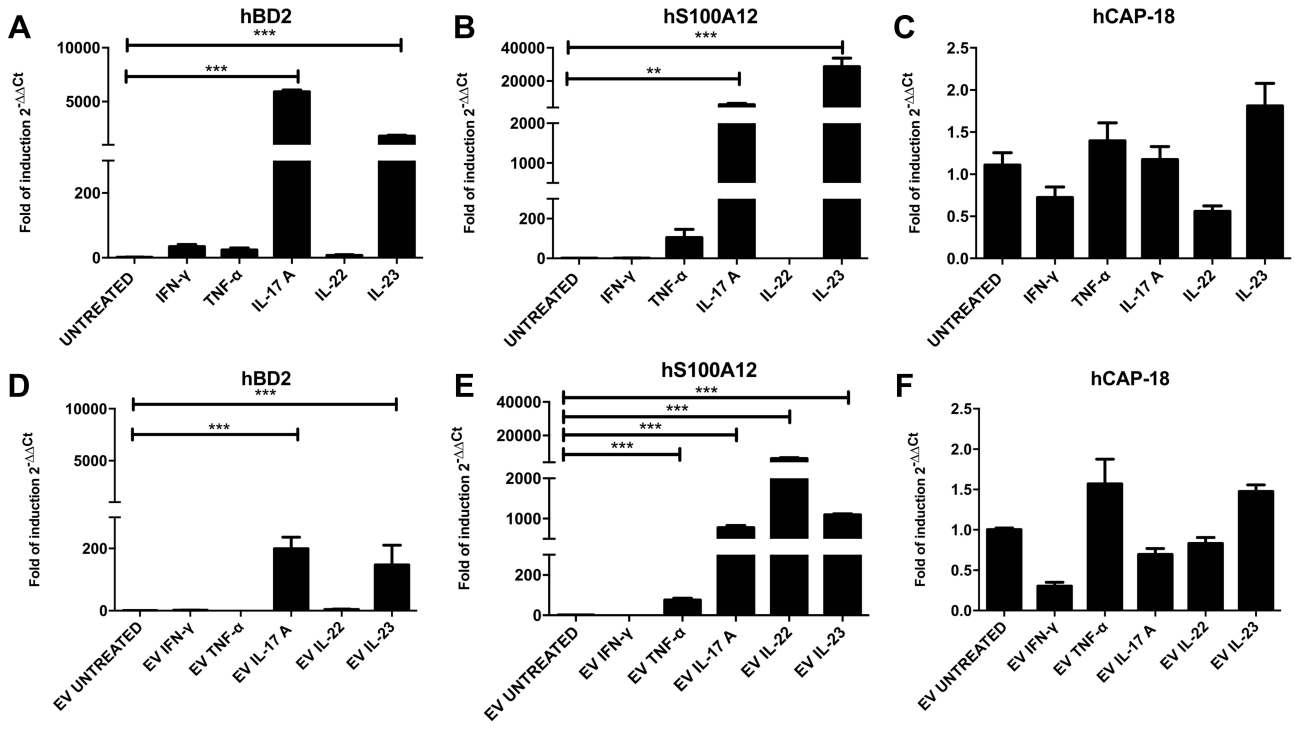

Figure 2 Different cytokine treatments alter mRNA expression of *hBD2*, *hS100A12* and *hCAP-18* in HaCaT cells and respective EVs. The mRNA expression of *hBD2*, *hS100A12* and *hCAP-18* was evaluated by Real Time RT-PCR in treated HaCaT cells and respective EVs isolated from supernatant. RNA was extracted, retro-transcribed and Real time RT-PCR performed on cellular (**A**–**C**) as well as EVs-associated (**D**–**F**) total cDNAs as described in Material and Methods section. Data represent means ± s.d. of three independent experiments. ***P ≤ 0.001; **P ≤ 0.01.

by inducing NETosis, we tested the ability of EVs derived from cytokine treated HaCaT cells to induce in vitro NETs formation. To this end, neutrophils were treated with EVs isolated from HaCaT cells previously stimulated with rIFN-γ, rTNF-α, rIL-17A, rIL-22 and rIL-23, then NETosis was evaluated by confocal microscopy. As depicted in Figure 3A, EVs derived from cells treated with all cytokines, but rIFNγ, were clearly able to trigger the release of extracellular traps identified as colocalization of DNA filaments (blue) and LL37 (green). In order to quantify the release of extracellular traps, the area covered by the extracellular complexes LL37/DNA was measured as described in Methods section and compared to the surface of untreated (non-netotic) cells (Figure 3B). EVs derived from keratinocytes stimulated with TNF-α, IL17A and IL-23 triggered a statistically significant release of NETs. On the contrary, IFN-γ and IL-22 were not able to induce a significant extracellular trap release.

EVs Isolated from HaCaT Cells Modulate Cell Migration

The characteristic lesion of psoriasis is due to the hyper-proliferation and the rapid migration of keratinocytes from the basal layer to the granular layer.[32] Recent reports showed that EVs are involved in cell proliferation and migration with a potential role in several diseases.[33,34] For the purpose of assessing the potential biological effect of EVs on acceptor cells, we have evaluated if EVs could modify the migration of HaCaT cells. Transwell migration assay showed that HaCaT cells incubated with EVs isolated from untreated cells migrated more than cells in control cultures (Figure 4). Conversely, HaCaT cells incubated with EVs derived from IL-17A treated cells migrated in a way superimposable to control cultures (Figure 4A). Interestingly enough, as shown for NET formation, IFN-γ acts in the opposite way, being HaCaT cells treated with EVs derived from IFN-γ treated cells able to migrate more than those treated with EVs isolated from control cultures (Figure 4B). Taken together, these results suggest that EVs can alter keratinocyte motility thereby contributing, together with proliferation induction, to the psoriatic lesion development.[35]

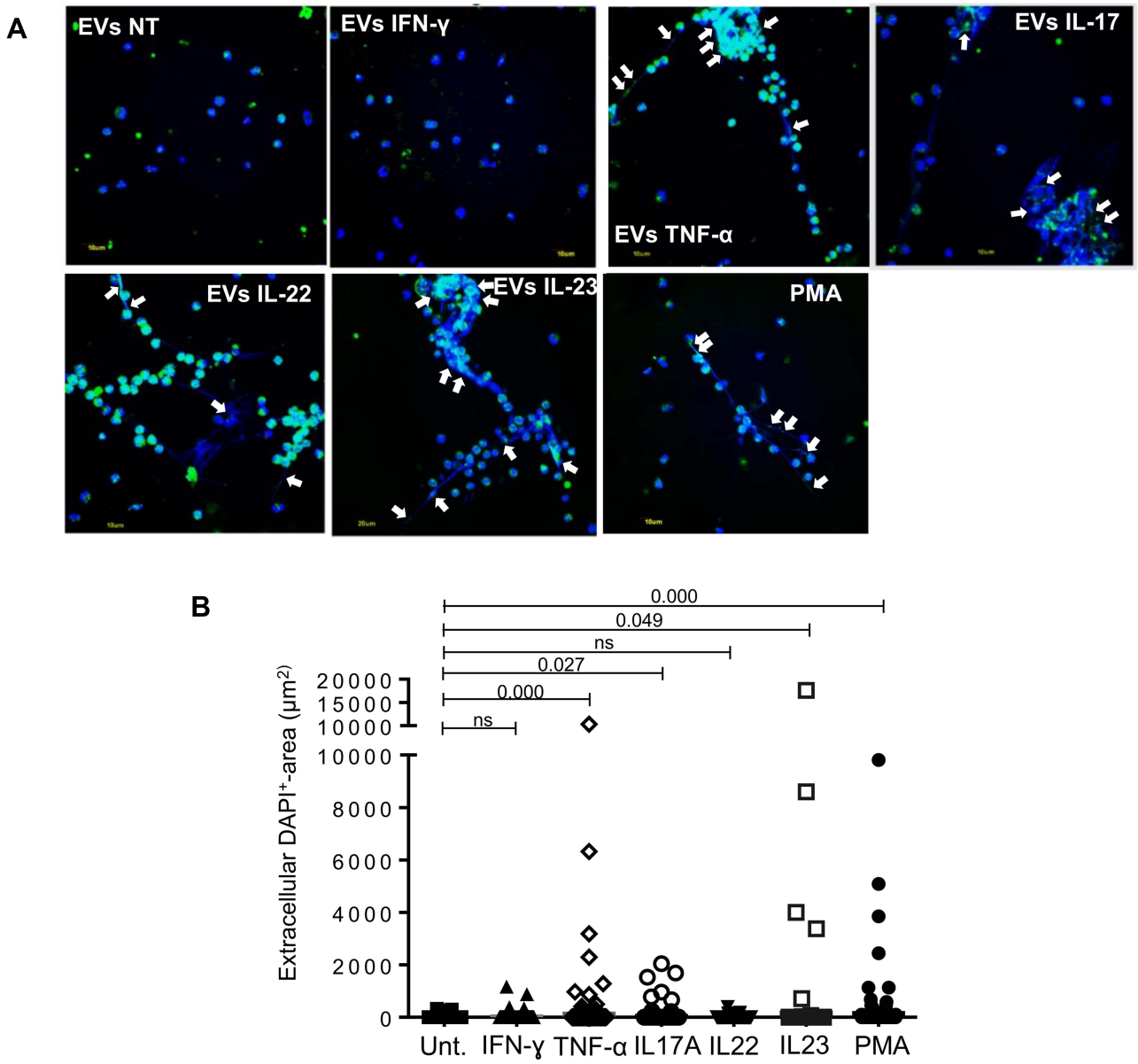

Figure 3 Keratinocyte-derived extracellular vesicles trigger netosis in vitro. (**A**) Healthy neutrophils were stimulated 3 hours with EV obtained from untreated HaCaTcells (NT) or previously treated with the indicated cytokines. Cells treated with the mitogen phorbol 12-myristate 13-acetate (PMA) is the positive control. Staining of the antimicrobial peptide LL37 was performed with AlexaFluor488-conjugated anti-human LL37 (green), and DNA filaments by a DNA dye (DAPI, blue). Scale bar is 10 µm. White arrows indicate extracellular LL37-DNA colocalizations. One experiment out of three is shown. (**B**) Neutrophils were treated as in (**A**). Extracellular traps were first identified as $DAPI^+$ (DNA) filaments associated with LL37, and then the area covered by DNA was quantified on three images per condition. Each dot represents the area covered by the extracellular DNA strand. One representative experiment out of two is shown. Results are shown as mean with standard error of the mean. P-values by Wilcoxon matched-pairs signed rank test.

HaCaT EVs Can Be Internalized by Condrosarcoma SW1353 and Synovial Sarcoma SW982 Cells

Since psoriatic arthritis is the most well recognized comorbidity of psoriasis, it was evaluated whether EVs derived from HaCaT cells could be internalized by condrosarcoma SW1353 and synovial sarcoma SW982 cells. To this purpose, HaCaT derived EVs were labeled with PKH67 fluorescent dye, then cellular uptake was assessed by flow cytometry, as described in Materials and Methods. As depicted in Figure 5 and in the corresponding cytograms (Supplementary Figure 1A), both cell types were able to internalize HaCaT EVs in a time-dependent manner, even if with different kinetics, with a maximum internalization rate at 6 hours.

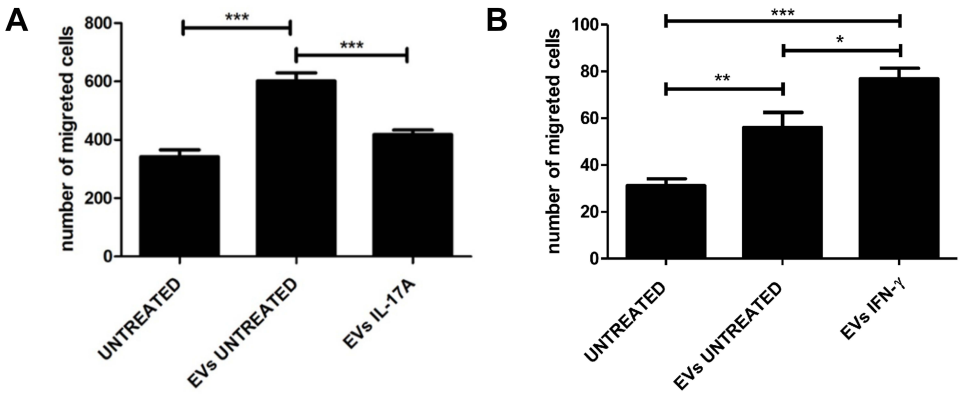

Figure 4 EVs isolated from rIL-17A or rIFNγ treated HaCaT cells can modulate HaCaT migration. (**A**) HaCat cells were treated with EVs isolated from HaCaT supernatant (EVs UNTREATED), with EVs derived from rIL-17A treated cells (EVs IL-17A) or left untreated (UNTREATED, used as control culture) as described in Materials and Methods. (**B**) HaCat cells were treated with EVs isolated from HaCaT supernatant (EVs UNTREATED), with EVs derived from rIFN-γ treated cells (EVs IFN-γ) or left untreated (UNTREATED). Data are presented as the mean ± SD of three independent experiments performed in triplicate. ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05.

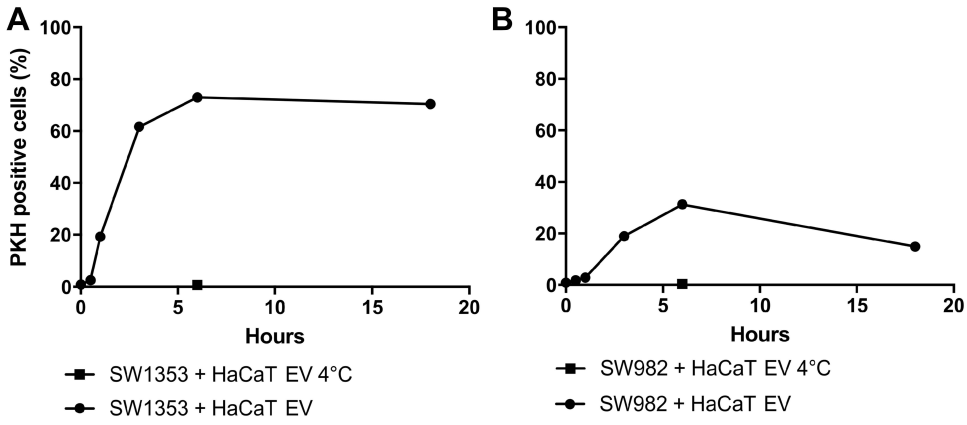

Figure 5 HaCaT EVs can be internalized by SW1353 and SW982 cell lines. EVs were isolated from HaCaT supernatant, stained with PHK67 and incubated with SW1353 (**A**) and SW982 (**B**) and cells for different time points. Cells fluorescence was assessed by FACS analysis. Incubation was performed at 37°C (black dots) or, as a negative control, for 6h at +4°C (black square). One representative experiment out of two is shown.

Psoriasis-Related EVs Isolated from HaCaT Cells Can Affect the Antimicrobial Peptide mRNAs Expression in Different Types of Acceptor Cells

Since EVs derived from keratinocytes treated with rIL-17A induced the expression of *hBD2* mRNA in homotypic acceptor HaCaT cells,[21] we tested the hypothesis that SW1353 and SW982 cells were also able to heterotypically express mRNAs carried by EVs derived from HaCaT cells. To verify this hypothesis, SW1353, SW982 and, as positive control, HaCaT cells were incubated with EVs, collected from HaCaT cells treated with psoriasis-related cytokine, then AMP mRNAs expression was evaluated in acceptor cell (+EV). In HaCaT cells, *hBD2* mRNA expression was upregulated by +EV IFN-γ and +EV IL-17A after 3 hours and with +EV IL-17A after 24 hours of treatment (Figure 6A, upper panel); *hS100A12* mRNA was upregulated by +EV IL-17A and +EV IL-23 and by +EV IFN-γ and +EV TNF-α after 3 and 24 hours of stimulation, respectively (Figure 6A, bottom panel); *hCAP-18* mRNA was increased by all EVs but EVs derived from TNF-α after 3 hours of stimulation and by +EV IFN-γ, +EV TNF-α after 24 hours (Figure 6A, middle panel). SW982 cells displayed an upregulated mRNA expression of *hBD2* when pulsed by +EV Untreated, +EV IL-17A, +EV TNF-α for 3 hours and an increment following +EV TNF-α stimulation for 24 hours (Figure 6B, top panel). *hCAP-18* mRNA expression is slightly upregulated into SW982 stimulated by all types of EVs at 3 hours whereas no induction was appreciable after a 24 hours treatment (Figure 6B, middle panel). Conversely, *hS100A12* gene is not expressed into SW982 cells. Finally, into SW1353 cell line the induction of *hBD2* expression was evident following +EV Untreated,

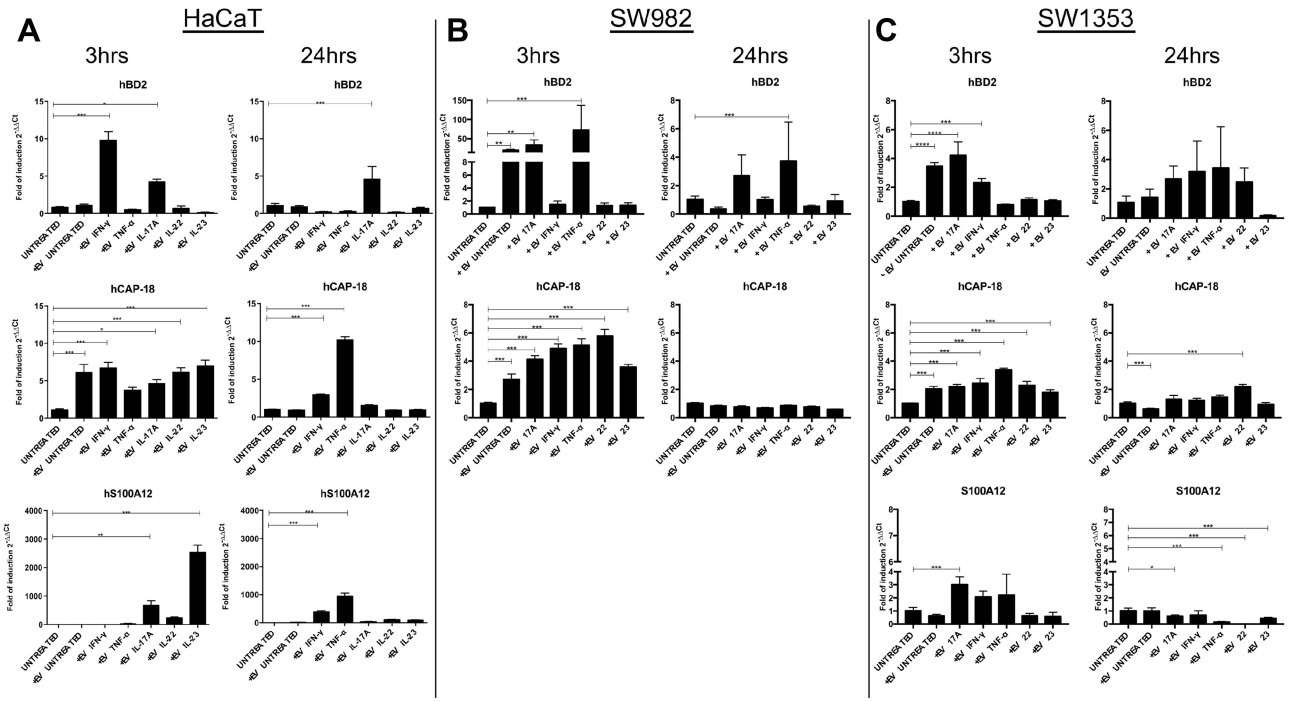

Figure 6 Different cytokine treatments modify the mRNA expression for *hCAP-18, hBD2* and *hS100A12* in HaCaT acceptor cells. The mRNA expression for *hCAP-18, hBD2* and *hS100A12* was evaluated in the EV-treated acceptor HaCaT (**A**), SW982 (**B**) and SW1353 cells (**C**) in comparison to untreated acceptor cells by Real Time RT-PCR. Cells were incubated for 3h and for 24h with EVs collected from supernatant of cytokine-treated HaCaT cells. Subsequently, RNA was extracted, retro-transcribed and Real time RT-PCR performed as described in Materials and Methods. Data represent means ± s.d. of three independent experiments. ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05.

+EV IL-17A and +EV IFN-γ stimulation for 3 hours (Figure 6C, top panel). *hCAP-18* mRNA expression was slightly downmodulated in +EV Untreated and upregulated in +EV IL-22 after 24h stimulation, whereas at 3h all the treatments induced slightly upregulation (Figure 6C, middle panel). *hS100A12* expression decreased when SW1353 cells were stimulated with +EV IL-17, +EV TNF-α, +EV IL-22 and +EV IL-23 for 24 hours.

Discussion

As in many inflammatory-associated pathological disorders ranging from autoimmune diseases to cancer,[36–39] psoriasis is characterized by an inflammatory microenvironment with specific inflammatory elements (ie, immune cells infiltrate, cytokines, chemokines, AMPs and DAMPs). To date, the role of EVs in psoriasis disease has not been deeply characterized yet. Previous data obtained in our laboratory showed that rIL-17A treatment was able to influence the release of EVs by HaCaT cells.[21] Furthermore, we also observed that *hBD2* mRNA is conveyed by EVs released by IL-17A treated HaCaT cells,[21] and human foreskin keratinocytes (HFK) (Supplementary Figure 2). Based on this evidence, we sought to extend our observations by verifying if other psoriatic-related cytokines were able to quantitatively and/or qualitatively tune the EVs release by using HaCaT cell line as an in vitro cellular model. Due to the importance of AMPs in the psoriasis pathogenesis,[40] we also evaluated the hypothesis that, beside *hBD2*, also mRNA for other AMPs could be conveyed by EVs.

As depicted in Figure 1, the treatment with IFN-γ, and even more, with IL-17A caused an increased EVs release compared to untreated cells or cells treated with other psoriasis-related cytokines. Interleukin-17 is the leading factor that, in cooperation with other inflammatory cytokines, settles the inflammatory psoriatic microenvironment.[41,42] On the other hand, it has been reported that IFN-γ acts as an activator of several antigen presenting cells (APCs) as monocytes, dendritic cells and endothelial cells as well as inhibitor of keratinocytes apoptosis.[43] EVs have also the ability to activate these cellular populations in other inflammatory settings. It has been, indeed, reported the ability of EVs isolated from oxidized low-density lipoprotein exposed endothelial cells to shift macrophage from M2 to pro-inflammatory M1 phenotype.[44] In addition, EVs produced by human keratinocytes induce the expression of pro-inflammatory genes in

Langerhans cells.[45] Based on these observations, it is tempting to speculate that EVs derived from IFN-γ treated keratinocytes cooperate with this cytokine to induce APCs activation.

In addition to their antimicrobial activity, AMPs have been proposed as DAMPs,[46] and they have been demonstrated to be abundantly expressed in psoriasis thereby contributing to the pathogenesis development.[47] Several studies suggest that EVs derived from stressed or injured tissues are able to carry DAMPs, thereby playing an important role in inflammation.[48] To verify if HaCaT cells treated with the psoriasis-related cytokines could modulate the AMPs mRNA expression and induce an increase of the mRNA cargo packed in related EVs, we analyzed mRNA expression into HaCaT derived EVs. We found a strong upregulation of *hBD2* and *S100A12* following IL-23 and IL-17A treatments (Figure 2D and E). *hS100A12* was strongly increased also by IL-22 (Figure 2E). IL-22 participates to psoriatic inflammation by acting directly on keratinocytes to promote cell stemness, by stimulating the secretion of IL-1β via the ROS-NLRP3-caspase-1 pathway, as well as by the upregulation of CD44, p63, and CD29 via NF-κB and MAPKs,[5,49–51] but there are not exhaustive data on S100A12 induction by IL-22. These observations corroborate the hypothesis that, beside the ability of IL-17A an IFN-γ to tune EVs release, psoriasis-related cytokines are able to manipulate EVs cargo in a selective manner to convey a specific message or to regulate intracellular process, thereby altering the phenotype and the function of recipient cells.[52] This could be the case when EVs are released in the lesional skin, then fueling the inflammatory microenvironment. To gain more insight into this "pro-inflammatory" EVs function we sought to test the hypothesis that EVs could play a role in the NETosis induction already observed in the psoriatic plaques.[27,53] The obtained results (Figure 3) clearly indicate that all the cytokines tested, with the only exception of IFN-γ and IL-22, induce the release of EVs able, in turn, to allow NETs formation. Again, this effect underlines how cytokines unable to induce a modification in the number of released EVs and/or in their mRNA cargo content (see prototypically TNFα) can alter in a yet uncharacterized way the features of EVs with the ultimate goal of settling/sustain the inflammatory microenvironment. Our results also confirmed those recently obtained by other groups using different experimental settings.[31] Additionally, our results might suggest a role of EVs in the neutrophilic activation in other neutrophilic diseases as Sweet syndrome, subcorneal pustular dermatosis and pyoderma gangrenosum.[54]

One of the most characteristic features of psoriasis is the exaggerated keratinocyte proliferation at the level of the psoriatic plaque.[55] We already demonstrated that EVs isolated from IL-17A treated HaCaT cells were unable to induce cell proliferation.[21] Here, we hypothesized that such a EVs could modulate the migration properties of keratinocytes. Interestingly enough, we report that EVs isolated from untreated cells induced migration of HaCaT cells in transwell migration assay, whereas EVs isolated from IL-17A treated cells were unable to do so (Figure 4A). It is tempting to speculate that, beside the deregulated keratinocyte proliferation, the reduction in the keratinocyte migration could contribute to the hypercellularity typically seen in the psoriatic lesion and that EVs released from the keratinocytes stimulated by the inflammatory microenvironment could play a role in this phenomenon. Another striking feature is the opposite migration behavior of keratinocytes treated with EVs derived from cells treated with IL-17A and IFNγ (compare Figure 4A and B). This result parallels the different ability of EVs IL-17A and EVs IFNγ to induce NETs formation in neutrophils even if, to date, we do not have any mechanistic explanation to justify these differences.

A recent report proved that extracellular vesicles isolated from blood of psoriatic patients carry IL-17A-exosome protein levels significantly higher in patients with moderate-to-severe psoriasis if compared with patients with mild psoriasis.[56] This suggests a possible role of EVs in the spreading of the disease once they gain access to the bloodstream, thereby contributing to the disease spreading and/or the appearance of comorbidities. To test if EVs could be hetero-tipically internalized by other than keratinocytes acceptor cells, we tested two different chondrocyte and synovial cell lines (ie, SW1353 and SW982, respectively). We resort to these cells with the purpose to verify a possible involvement of keratinocyte-derived EVs in the settlement of psoriatic arthritis. Indeed, these cell lines were already used as an in vitro cellular model resembling osteoarthritis and rheumatoid arthritis, respectively.[57,58] We verified that both SW1353 and SW982 internalized EVs isolated from untreated HaCaT cells, even if with different kinetics (Figure 5). SW1353 cells internalize EVs in a superimposable way to HaCaT acceptor cells (compare Figure 5A with Supplementary Figure 1B) whereas SW982 cells display a reduced internalization rate. No differences in the internalization rate were recorded when EVs isolated from untreated or cytokine-treated keratinocytes were used (data not shown). Collectively, these data demonstrate that EVs internalization can take place with the same or different kinetics irrespective of his homo- and

heterotypic nature (ie, internalization of EVs by acceptors of the same lineage of donor cells or from different lineage, respectively). However, the molecular details that could explain the difference in the internalization rate remain elusive.

Our previous data demonstrated that EVs isolated from HaCaT cells are able to induce the expression of *hBD2* mRNA on homotypic acceptor cells, due to their ability to specifically carry IL-17A.[21] Here we demonstrated that, beside keratinocyte-to-keratinocyte communication, EVs also affect transcriptional profile of other cell lineages. In particular, we observed that *hBD2, hCAP-18* and *S100A12* mRNA are modulated by treatment with EV derived from psoriasis-related cytokines stimulated HaCaT not only in homotypic cells (Figure 6A), but also in SW1353 and SW982 cells (Figure 6B and C). Interestingly, some AMP's mRNAs (see, for example, CAP-18 mRNA at 3hrs, Figure 6A–C, middle panels) were upregulated in acceptor cells even by EVs isolated from untreated HaCaT cells, or from EVs that, we reported, do not carry the relevant mRNAs in their cargo (ie, CAP-18 mRNA, see Figure 2F). This implies two sets of reflections: first, as we already demonstrated from *hBD2*,[21] these upregulation are probably the result of a *de novo* mRNA synthesis from the endogenous gene encoded from the acceptor cell, rather than a transfer from the EVs cargo. Second, the cell signaling possibly activated by EVs responsible of mRNA transcription is triggered by a constitutively present cargo element, being elicited also by EVs isolated from untreated cells. These data corroborate the observation of the capability of cultured synoviocytes and chondrocytes to increase *hBD2* expression following exposition to bacterial components or some inflammatory cytokines, respectively.[59,60] Also, *CAP-18* expression in synovial membrane samples from patients with pyogenic arthritis, osteoarthritis or rheumatoid arthritis, has been demonstrated.[61] *S100A12* levels in synovial fluid is associated with clinical severity in patients with osteoarthritis,[62] and is used as a marker for systemic-onset juvenile idiopathic arthritis.[63] Collectively, our results supported the idea that EVs released by lesional keratinocytes could fueling psoriatic comorbidities once released into the bloodstream.

Conclusion

The presented data indicate that: i) psoriasis-associated cytokines (ie, IL-17A and IFNγ) can modify the number of released EVs as well as their cargo content; ii) EVs can carry mRNAs for specific antimicrobial peptides and iii) EVs could be internalized by different cell types (ie, neutrophils, chondrocytes and synovial cells), thereby modulating effector functions (ie, NETosis and gene expression). Collectively, the reported results highlight the role of EVs in the composition of psoriasis-associated secretome and microenvironment also suggesting the EVs involvement in the disease mediators spreading and in the possibly associated comorbidities. One of the main limitations of this study is the use of an in vitro "reconstructed" cellular system to simulate the psoriatic cellular microenvironment. More studies, also involving EVs specimens collected from psoriasis patients, are needed not only to confirm our data but also to deepen the mechanisms required for the cargo upload into EVs during psoriasis development and to better understand the role of EVs into disease spreading and comorbidity development.

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Disclosure

The authors report no conflicts of interest in this work.

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